Phosphorus supply drives rapid turnover of membrane phospholipids in the diatom *Thalassiosira pseudonana*.

Patrick Martin,[a,*] Benjamin A. S. Van Mooy,[b] Abigail Heithoff,[c] Sonya T. Dyhrman[c]

[a]National Oceanography Centre Southampton, European Way, Southampton, UK

[b]Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, USA

[c]Biology Department, Woods Hole Oceanographic Institution, Woods Hole, USA

[*]Corresponding author: patrick.martin@noc.soton.ac.uk, Tel: +44 (0)2380 596336, Fax: +44 (0)2380 596247

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Abstract

In low phosphorus (P) marine systems phytoplankton replace membrane phospholipids with non-phosphorus lipids, but it is not known how rapidly this substitution occurs. Here, when cells of the model diatom *Thalassiosira pseudonana* were transferred from P-replete medium to P-free medium, the phospholipid content of the cells rapidly declined within 48 h from 45±0.9% to 21±4.5% of total membrane lipids; the difference was made up by non-phosphorus lipids. Conversely, when P-limited *T. pseudonana* were resupplied with P, cells reduced the percentage of their total membrane lipid contributed by a non-phosphorus lipid from 43±1.5% to 7.3±0.9% within 24 h, while the contribution by phospholipids rose from 2.2±0.1% to 44±3%. This dynamic phospholipid reservoir contained sufficient P to synthesize multiple haploid genomes, suggesting that phospholipid turnover could be an important P source for cells. Field observations of phytoplankton lipid content may thus reflect short-term changes in P supply and cellular physiology, rather than simply long-term adjustment to the environment.

Phosphate is chronically scarce in oligotrophic oceans (Krom et al., 1991; Karl et al., 1997; Wu et al., 2000), and phytoplankton appear to posses highly effective physiological mechanisms to reduce their phosphorus (P) quota in these environments (Twining et al., 2010). For example, all phytoplankton groups examined to date, both in culture and in situ, substitute non-phosphorus (sulfur- and nitrogen-containing) lipids for phospholipids in their membranes under low-P conditions (Van Mooy et al., 2009). Such lipid substitution in bacteria (Minnikin et al., 1974; Benning et al., 1995) can take place within hours (Zavaleta-Pastor et al., 2010). However, it is not known how rapidly phytoplankton remodel their membranes, and whether field observations (Van Mooy et al., 2009) reflect long-term adjustment to the environment, or an immediate cellular response to low-P conditions.

The diatom *Thalassiosira pseudonana*, and other eukaryotic phytoplankton, contain the phospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE). Under low-P conditions, *T. pseudonana* substitutes PC with the betaine lipid diacylglycerylcarboxyhydroxymethylcholine (DGCC), and PG with the sulfolipid sulfoquinovosyldiacylglycerol (SQDG) (Van Mooy et al., 2009).

Two culture experiments were conducted to test how rapidly lipid substitution occurs in *T. pseudonana* upon changes in phosphate concentration. In the first experiment, *T. pseudonana* CCMP1335 was grown in P-replete medium (in triplicate, Supplementary Methods) until early log-phase. Cells were then gently filtered onto 0.2 μm polycarbonate membranes and resuspended in either phosphate-free (-P) or phosphate-replete (+P, 36 μmol L^{-1} phosphate) medium. However, as the -P cultures contained 0.5±0.2 μmol L^{-1} phosphate at 0 h, representing a carryover of the original culture medium on the order of 1%, cells did not experience limiting levels of P instantaneously. Samples for intact polar lipids (IPLs) and cell counts were taken over 48 h (Supplementary Methods).

The cells responded quickly after transfer, and grew equally well in the -P and +P treatments over 48 h (Figure 1a). However, despite the minor carryover of P in the culture medium, the DGCC:PC ratio increased sharply in the -P treatments after 24 h, relative to the +P controls, indicating lipid substitution in response to low-P conditions (Figure 1b, Supplementary Figure 1). Similarly, the SQDG:PG ratio rose in the -P compared to the +P treatments (Figure 1c). The molar percentage contribution by phospholipids to total IPLs fell from 45±0.9% at 0 h to 21±4.5% at 48 h in the -P cultures (Figure 1d). Concomitantly, the contribution by DGCC rose from undetectable levels to 11.5±1.7%, and the contribution by SQDG from 38±2% to 52±6% (Supplementary Figure 1). The contribution by the lipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) did not differ between treatments (Supplementary Figure 1). This contrasts with higher plants, which have been shown to substitute PC with DGDG (Tjellström et al., 2008).

The per-cell content of the phospholipids PC, PG, and PE dropped from 1.8±0.2 fmol $cell^{-1}$ at 6 h to 0.92±0.12 fmol $cell^{-1}$ (Figure 1e). Moreover, the amount of phospholipid per mL of culture medium decreased by 160±30 pmol mL^{-1} in the -P treatments between 24 h and 48 h (Supplementary Figure 1), indicating that the per-cell shift in phospholipids was caused by net breakdown of these molecules. This breakdown of phospholipids represented a quantity of P equivalent to 3.0±0.6 x 10^8 atoms P per cell for the new cells that grew during the 24 h to 48 h time period. As *T. pseudonana* contains 7.2 x 10^7 P atoms per haploid genome (von Dassow et al., 2008; Supplementary Methods), this is equivalent to the amount of P needed for each cell to synthesize about 4 haploid genomes. Clearly, the phospholipid pool represents a significant amount of P that is rapidly mobilized in response to low-P conditions.

The relatively constant PE:PC ratio (Figure 1f) underscores that the breakdown rates of these two lipids were relatively equal. This supports the finding of Riekhof et al. (2005) that betaine lipid

84 synthesis is linked to the cleavage of phospholipid headgroups (e.g. via indiscriminant phospholipase
85 C activity).
86
87 To test the response-time of P-limited *T. pseudonana* to P resupply, triplicate flasks of *T. pseudonana*
88 were grown (Supplementary Methods) until P-limited, and then resupplied with 36 μmol L^{-1} phosphate
89 (Refeed treatment), and tracked relative to triplicate P-replete (+P) and P-limited (-P) controls. The
90 cells in the Refeed cultures resumed growth within 24 h after phosphate addition (Figure 2a). The
91 DGCC:PC ratio in the Refeed cultures fell from an initial value of 33.7±0.4 to 1.7±0.3 at 12 h, with a
92 discernable change within the first hour (Figure 2b), and this was driven both by the breakdown of
93 DGCC and the synthesis of new PC. DGCC per cell decreased after 5 h (Supplementary Figure 2),
94 and the molar percentage of IPL contributed by DGCC fell from 43±1.5% initially to 7.3±0.9% at 24 h
95 (Supplementary Figure 2). The SQDG:PG ratio differed dramatically between +P and -P cultures, and
96 in the Refeed cultures it dropped to values identical to the +P cultures by 24 h (Figure 2c). Change in
97 the SQDG:PG ratio was driven by changes in PG content; the percentage contribution by SQDG (and
98 by MGDG and DGDG) remained largely constant and identical between treatments (Supplementary
99 Figure 2). The PE:PC ratio spiked sharply in the first 12 h (Figure 2f), which is consistent with the
100 activation of a synthesis pathway where PC is synthesized from PE as has been observed in yeast (but
101 not in plants; Birner and Daum, 2003).
102
103 In the Refeed treatments, the percentage contribution by phospholipids to total IPLs increased from
104 2.2±0.1% at 0 h to 44±3% at 24 h (Figure 2d). The overall phospholipid content increased by 1.2±0.1
105 fmol $cell^{-1}$, or 7.4±0.5 x 10^8 atoms P $cell^{-1}$, which is enough P to synthesize ten haploid genomes
106 (Figure 2e). Overall, *T. pseudonana* responds rapidly to P resupply, increasing phospholipid synthesis
107 and degrading DGCC. DGCC contains nitrogen, and there may be a physiological benefit to actively
108 recycling DGCC.
109

These data demonstrate that the IPL composition of *T. pseudonana* is dynamic and changes within one cell division or less in response to changes in external P concentrations. The breakdown of phospholipids appears to liberate a significant quantity of P, enough for the synthesis of several genomes. Likewise, cells can sequester a significant amount of P in phospholipids when P concentrations increase suddenly. Recycling of P from phospholipids may be able to support several cell divisions in low-P conditions. This suggests that recent observations of elevated betaine lipid:PC and SQDG:PG ratios in low-P marine systems (Van Mooy et al., 2006; 2009) at least partly reflect a short-term physiological response to low P. Most marine phytoplankton do appear to have inherently low phospholipid contents, even when P is replete (Van Mooy and Fredericks, 2010; Van Mooy et al., 2009). While it has not yet been tested whether P enrichment of low-P ocean samples leads to changes in whole-community lipid composition, at least one phytoplankton taxon, the ubiquitous cyanobacterium *Synechococcus* (which undertakes SQDG–PG substitution; Van Mooy et al, 2009), can increase its total cellular P-quota upon short-term P supply by mesoscale eddies (Twining et al., 2010). Our data indicate that membrane remodeling may be important for such a response, and that membrane remodeling is an important and dynamic process for phytoplankton in the sea.

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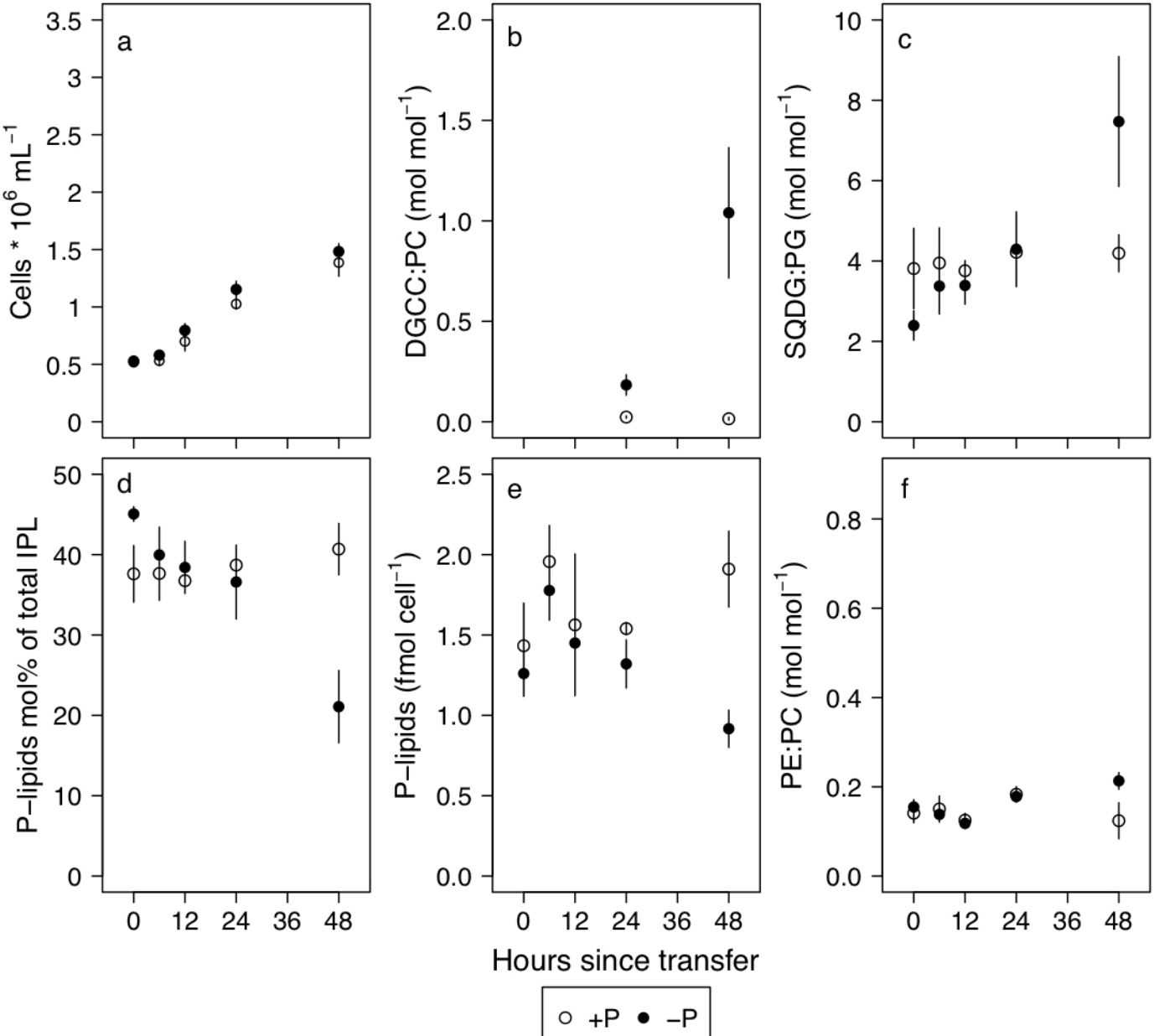
169 Figure Legends

170

171 Figure 1. A time course of parameters in an experiment where P-replete *T. pseudonana* cells were
172 harvested and transferred into either P replete (+P) or P-free (-P) medium. (a) Cell growth in +P and -P
173 treatments; (b) DGCC:PC ratio; (c) SQDG:PG ratio; (d) combined molar percentage of total IPL
174 contributed by the phospholipids PC, PG, and PE; (e) combined content of the three phospholipids PC,
175 PG, and PE per cell; (f) PE:PC ratio. All data are treatment mean ± 1 standard deviation of $n = 3$.

176

177 Figure 2. A time course of parameters in an experiment where P-limited *T. pseudonana* cells (-P) were
178 resupplied with phosphate (Refeed) and compared to replete (+P) treatments. (a) Cell growth curve.
179 The solid horizontal line between Days 10 and 11 indicates the 24 h period over which lipids were
180 sampled, starting from the time of phosphate resupply. (b)–(f) share a common *x*-axis corresponding to
181 the horizontal line shown in (a). (b) DGCC:PC ratio; (c) SQDG:PG ratio; (d) combined molar
182 percentage of total IPL contributed by PC, PG, and PE; (e) combined per-cell content of PC, PG, and
183 PE; (f) PE:PC ratio. All data are mean ± 1 standard deviation of $n = 3$.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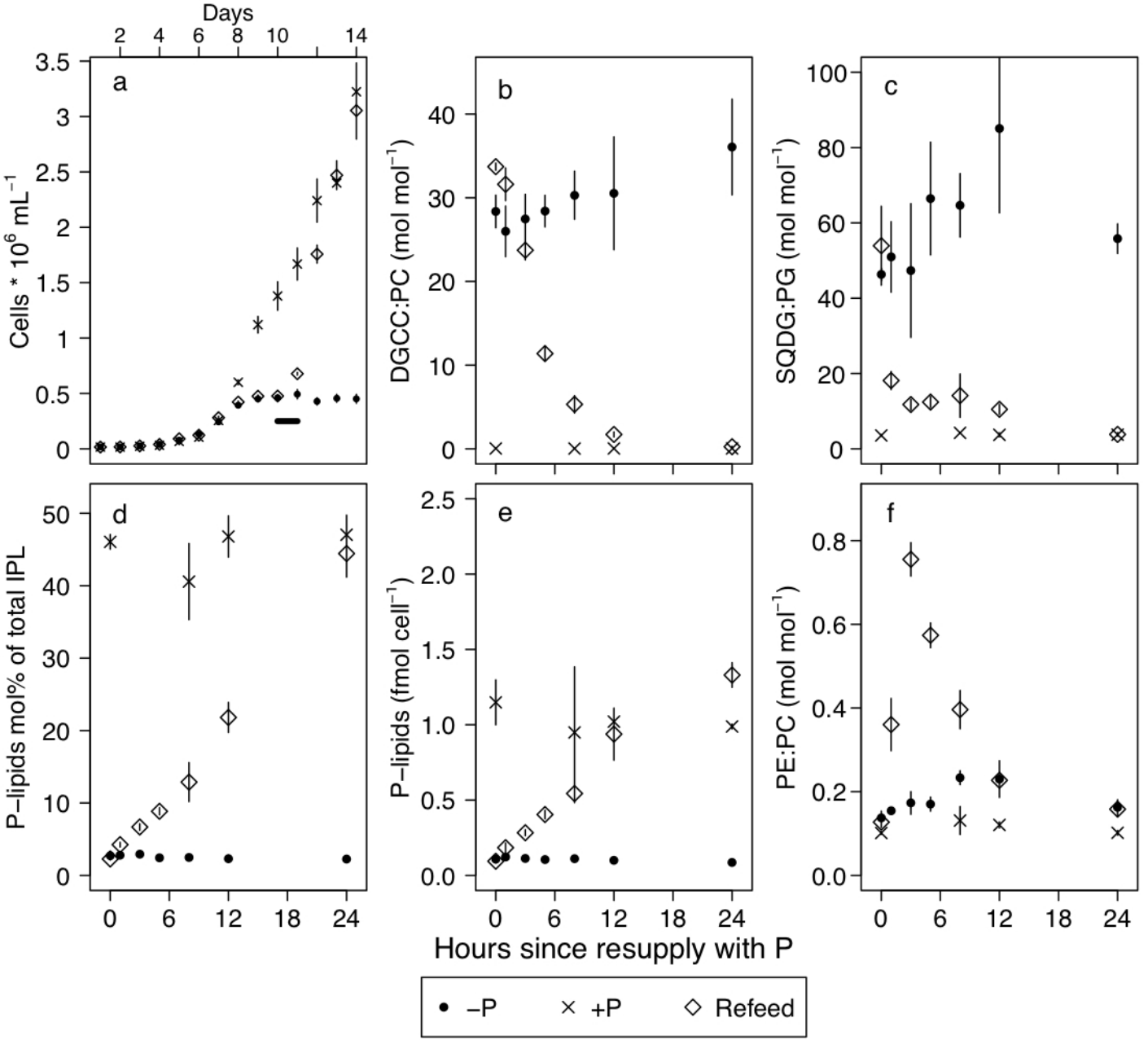
Cells * 10^6 mL^-1
DGCC:PC (mol mol^-1)
SQDG:PG (mol mol^-1)
P–lipids mol% of total IPL
P–lipids (fmol cell^-1)
PE:PC (mol mol^-1)
Hours since transfer
+P
–P


Days
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4
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a
Cells * 10^6 mL^-1
b
DGCC:PC (mol mol^-1)
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SQDG:PG (mol mol^-1)
d
P−lipids mol% of total IPL
e
P−lipids (fmol cell^-1)
f
PE:PC (mol mol^-1)
Hours since resupply with P
−P
+P
Refeed

Supplementary Information to Martin et al.: Phosphorus supply drives rapid turnover of membrane phospholipids in the diatom *Thalassiosira pseudonana*

Supplementary Methods

Cultures

Axenic *Thalassiosira pseudonana* CCMP 1335 was grown for both experiments in borosilicate flasks in f/2 medium (Guillard, 1975) made with a local sea water base or artificial sea water. In the first experiment, medium was made with artificial seawater (Kester et al., 1967) either without added phosphate (-P treatment), or with 36 µM phosphate (+P treatment). In the second experiment, local seawater base was used, with phosphate either reduced to 1 µM to induce P limitation (-P and Refeed treatments), or at 36 µM (+P treatment). All cultures were kept at 18ºC on a 14:10 light:dark cycle at 113 µmol quanta m^{-2} s^{-1}. The phosphate concentrations in the growth media were not manipulated during the experimental period, apart from in the Refeed treatments. Growth was monitored by direct cell counts on a haemocytometer or by relative fluorescence on an Aquafluor hand-held fluorometer. The fluorescence per cell was the same in both the -P and +P treatments, and cell numbers were calculated from relative fluorescence using an empirically established relationship derived from *T. pseudonana* CCMP1335 cultures (cells mL^{-1} = 826130 ± 25544 * fluorescence, r^2 = 0.985),

Lipids

Lipid samples (5–20 mL) were filtered onto precombusted GF/F filters, flash-frozen in liquid nitrogen, and stored at -80ºC for less than one month until analysis. Intact polar lipids (IPLs) were extracted according to a protocol modified from Bligh and Dyer (1959), using phosphate buffer (PBS), methanol, and dichloromethane as solvents, and quantified using high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC ESI-MS). The synthetic lipid

dinitrophenylphosphoethanolamine (Avanti Polar Lipids, Alabaster, USA) was added during extraction as a yield monitor. Commercially-available authentic lipid standards from Avanti Polar Lipids Inc. (Alabaster, USA) were used to quantify phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and from Matreya LLC (Pleasant Gap, USA) to quantify monogalactosyldiacylglycerol (MGDG), and digalactosyldiacylglycerol (DGDG). Authentic standards for sulfoquinovosyldiacylglycerol (SQDG) and diacylglycerylcarboxyhydroxymethylcholine (DGCC) were purified from cultures of *Synechococcus* WH8102 and of *Thalassiosira pseudonana*, respectively, using preparative HPLC, and quantified gravimetrically.

For both experiments, calculations of lipid-stocks, lipid ratios, and P atoms per cell were made first for each replicate culture, and then averaged between treatments. The paper presents the mean results.

Genome-P stock

The haploid genome size of *T. pseudonana* CCMP1335 is approximately 36 MBp, including organelle DNA (von Dassow et al., 2008). As each basepair is joined by two phosphate moieties to the next basepair, we calculate that the haploid genome contains $2 \times 36 \times 10^6 = 7.2 \times 10^7$ atoms of P.

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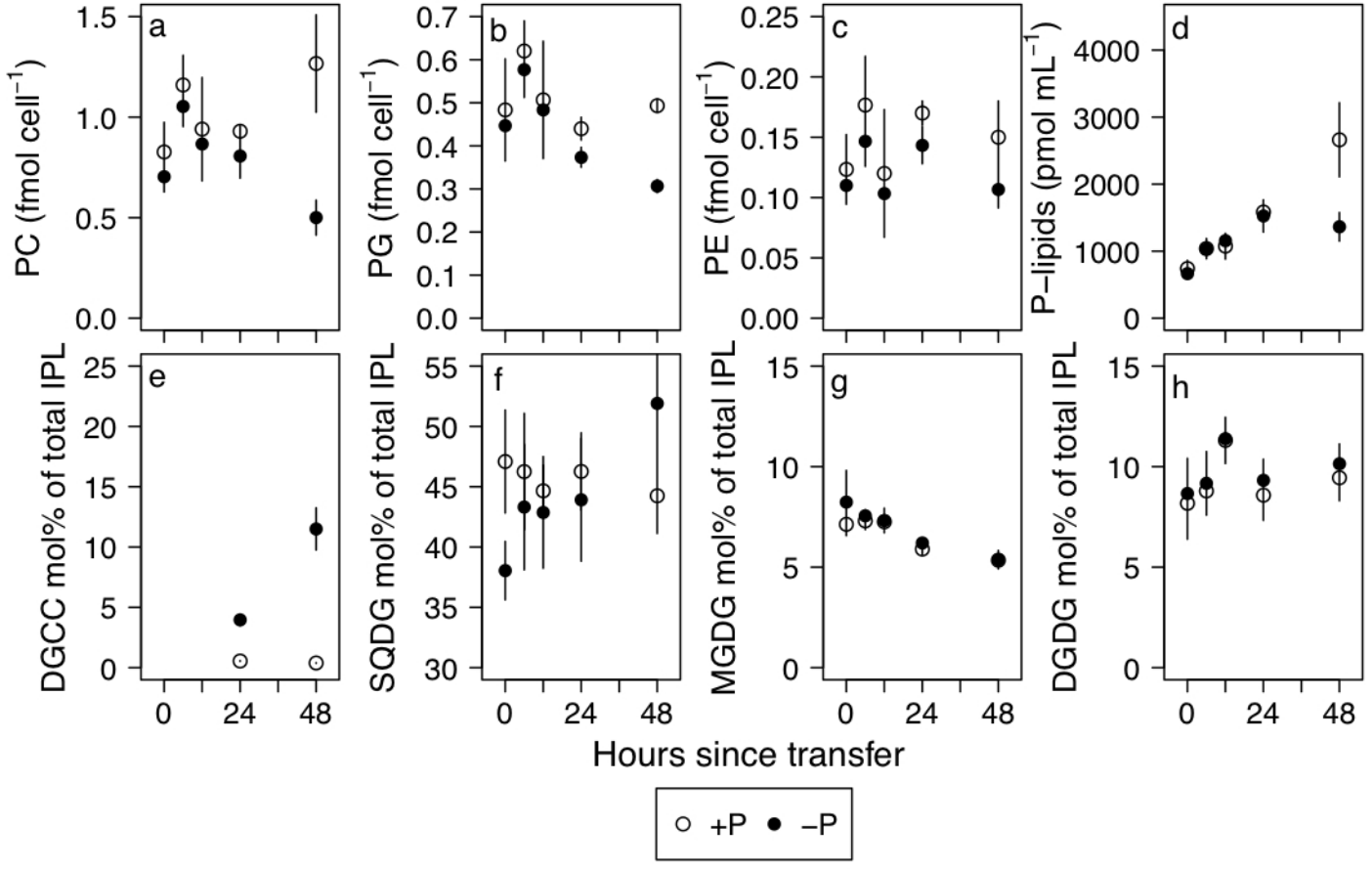
Supplementary Figure Legends

Supplementary Figure 1. A time course of parameters in an experiment where P-replete *T. pseudonana* cells were harvested and transferred into either P-replete (+P) or P-free (-P) medium. Per-cell content of (a) PC; (b) PG; and (c) PE. (d) Concentration of total phospholipids (PC+PG+PE), expressed per mL, indicating the breakdown of phospholipids between 24 and 48 h. Molar percentage of total IPL contributed by (e) DGCC; (f) SQDG; (g) MGDG; (h) DGDG (note different *y*-axis scales). All data are treatment mean ± 1 standard deviation of $n = 3$.

Supplementary Figure 2. A time course of parameters in an experiment where P-limited *T. pseudonana* cells (-P) were resupplied with phosphate (Refeed) and compared to replete (+P) treatments. Per-cell content of (a) PC; (b) PG; (c) PE; and (d) DGCC. Molar percentage of total IPL contributed by (e) DGCC; (f) SQDG; (g) MGDG; (h) DGDG (note different *y*-axis scales). All data are treatment mean ± 1 standard deviation of $n = 3$.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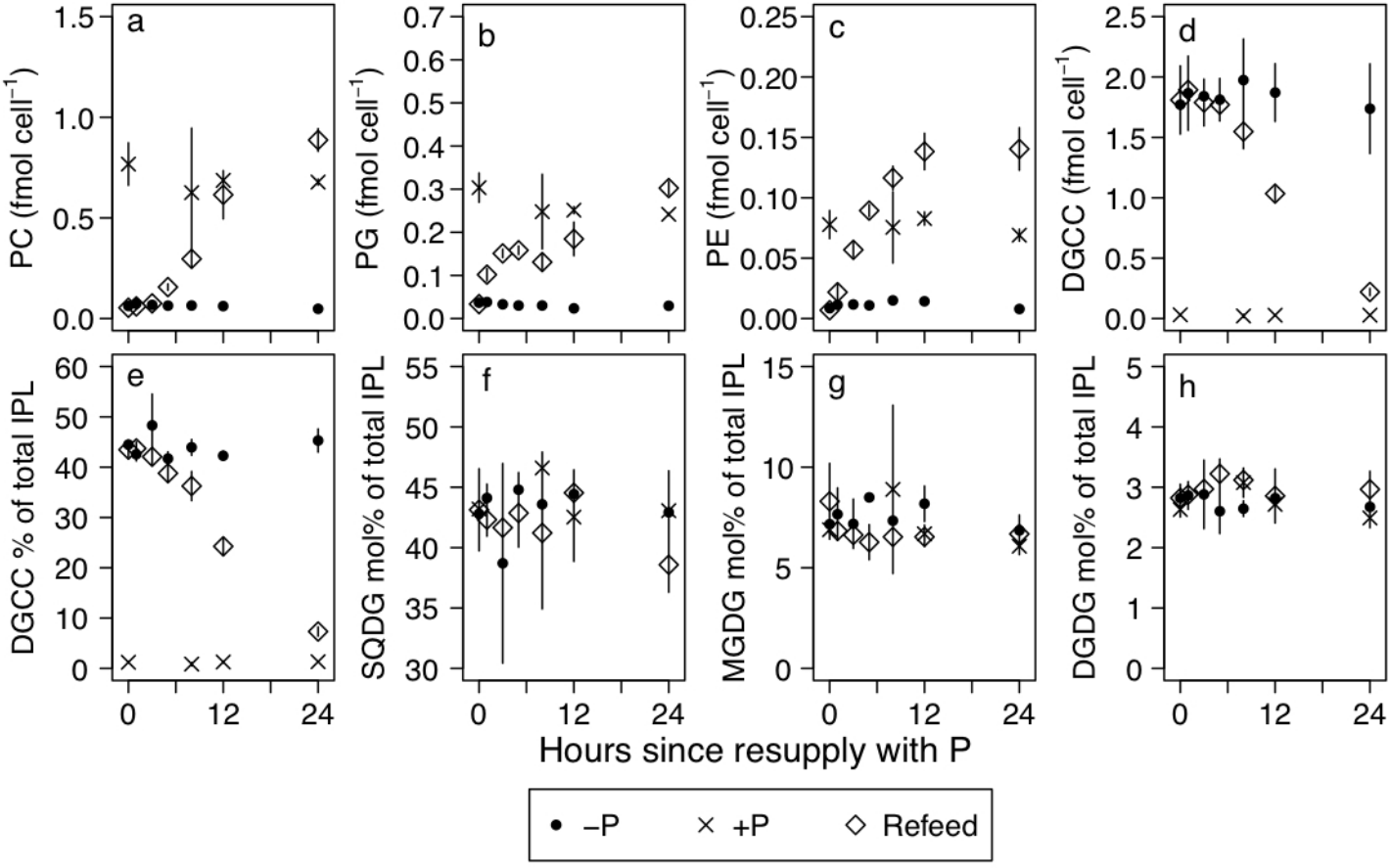
PC (fmol cell^{-1})
PG (fmol cell^{-1})
PE (fmol cell^{-1})
P−lipids (pmol mL^{-1})
DGCC mol% of total IPL
SQDG mol% of total IPL
MGDG mol% of total IPL
DGDG mol% of total IPL
Hours since transfer
+P
−P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PC (fmol cell^{-1})
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DGCC % of total IPL
SQDG mol% of total IPL
MGDG mol% of total IPL
DGDG mol% of total IPL
Hours since resupply with P
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Refeed